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STRUCTURE AND EXPRESSION OF GENES FOR FLAVIVIRUS IMMUNOGENS

Annual Summary Report

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Progress during the reporting period included: i) expression of a DEN-1 virus neutralizing epitope in E. coli ii) expression of DEN-1 structural proteins and 80% of the NS1 protein by a recombinant baculovirus iii) extension of the DEN-1 sequence analysis into the NS5 coding region and iv) development of an assay to detect DEN-1 RNA by non-radioactive means.

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FOREWORD

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals", prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

For the protection of human subjects, the investigator(s) have adhered to policies of applicable Federal Law 45CFR46.

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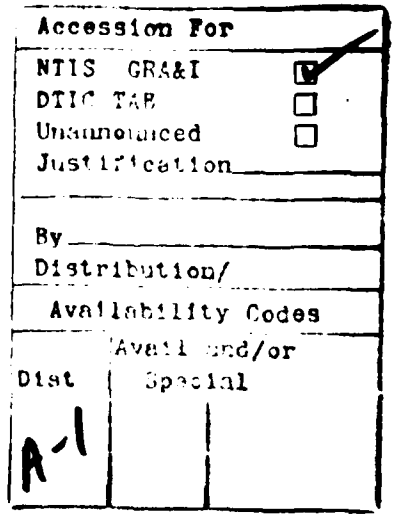

Annual Report - DAMD 17-86-C-6156
15 June 1988 - 14 June 1989

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Dr. Zhang Yong-he	Guest Scholar+
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* Designates contract employees

Mahidol University, Bangkok, Thailand
2/88 - 2/89
Salary support provided by Fogarty Fellowship

+ Chinese Academy of Medical Sciences
10/87 - 10/88
Salary support provided by grant from W.H.O. t o Co-PIs

B. Project Aims:

Stated objectives for the current project period include:

1) To explore the potential for developing dengue 1 (DEN-1) and Japanese encephilitis virus (JEV) subunit vaccines. Emphasis has been divided between *E. coli* host-vector expression systems and production of recombinant E and NS1 immunogens in animal and insect host cells.

2) To map and define at the molecular level the structures of the immunologically important domains of the E and NS1 proteins.

3) To develop cDNA and RNA probes for use in research and clinical diagnosis.

4) To extend the sequence analysis of the JEV and DEN-1 genomes.

C. Program

1) Recombinant E and NS1 Immunogens

Expression and evaluation of recombinant immunogens produced in E. coli

Three DEN-1 E domain-II recombinant proteins have been expressed in *E. coli*. The first of these delta-34 (spanning amino acids 238-413 of the DEN-1 E protein) has been described in an earlier reports. Previously, we reported expression of this protein using the plasmid vector pRR1 (expression was driven by the P_L promoter of lambda) and purification by acid extraction and column chromatography. We have now cloned the coding region for this protein and two additional proteins, delta-34 cys-to-ser (identical to delta-34 except cysteine285 was modified to serine to prevent it from interfering with the cysteine302:cysteine334 disulfide bond) and delta-31 (spanning amino acids 293-413 of the DEN-1 E protein) into the T7 RNA polymerase expression vector, pET3c. This vestor allows inducible expressions of recombinant proteins using the T7 RNA polymerase. These proteins were expressed at high levels in this system, i.e. approximately 20% of total cell protein. They could be visualized by coomassie blue staining and by immunoblotting using the virus neutralizing antibody 9D12. It is interesting to note that both delta-31 and delta-34 cys-to-ser which could not be detectably expressed by the pRR1 expression vector are expressed efficiently by this system.

Polyclonal antibodies have been produced in animals immunized with either of both forms of delta-34 and been tested for their ability to react with authentic DEN-1 E antigen. These antisera were found to react strongly with DEN-1 antigen as observed by ELISA and immunoblotting procedures. However, this binding was not sulfhydral-dependent and these antisera could not immunoprecipitate the recombinant antigens.

Expression of DEN-1 immunogens in yeast

Expression of Den-1 structural proteins was attempted with the yeast *Saccharomyces cerevisiae* with the idea of testing this system which could provide an enviroment more conducive to proper protein folding. The two constructs tested were fused to the galactose-inducible GAL1 promoter. Disappointingly, no evidence of expression of the recombinant proteins was obtained.

Expression and evaluation of recombinant immunogens from baculoviruses

Three recombinant baculoviruses which express recombinant DEN-1 proteins have been isolated. The best characterized of these, BR-7, contains the coding sequences for C, prM, M, E and 80% of NS1. Fixed cells infected with BR-7 were positive in an immunofluorescence assay using an anti-DEN-1 hyperrimmune mouse ascites fluid (HMAF), an anti-NS1 HMAF and monoclonal antibodies 9D12 and 7E11 which are directed against E and NS1, respectively. Immunoblotting performed on BR-7-infected cell extracts using the 9D12 antibody revealed that the recombinant E protein produced by these cells comigrates with E from DEN-1-infected mosquito cells. A truncated form of NS1 was also detected in these extracts using the 7E11 antibody. All of the antisera tested identified proteins which were larger than E or NS1 in the BR-7-infected cell extract. These are presumed to be either unprocessed polyprotein or partially or improperly processed polyproteins.

Polyclonal antisera produced in four animals immunized with baculoviruses-infected cell extracts were evaluated by immunoblotting. One of these antisera were found to react with NS1 from DEN-1-infected C6/36 cell extracts suggesting that DEN-1 proteins produced in this system are capable of illiciting an immune response with recognizes authentic DEN-1 proteins.

Detection of dengue virus RNA by non-radioactive methods

This work is being conducted by a visiting collegue, Dr. W. Attatippaholkun, Clinical Instructor from Mahidol University in Bangkok, Thailand. Dr. Attatippaholkun is supported by a Fogarty Fellowship which provides support for a portion of the supplies required for her work; the remaining supplies are purchased from the current contract and a W.H.O. grant to the co-PIs. The aim of this project is to carry out side-by-side comparisons of the various non-radioactive methods for detecting RNA or DNA using specific RNA or DNA probes.

Results have been obtained from side-by-side comparison of DEN-1 probes tagged with biotin by three different enzymatic methods: i) *in vitro* transcription of cDNA using T7 and T3 RNA polymerases; ii) nick-translation of cDNA fragments; and iii) random primer labeling of cDNA. Using a dot blot assay it was show that the RNA probes were the most sensitive, followed by random primer labeled DNA and nick-translated DNA, in that order.

Hydridization analyses were performed using the labeled RNA and a variety of target RNAs including those from uninfected and DEN-1 infected C6/36 cells and a DEN-1 virion preparation. Results to date have shown that less than 5 pg of DEN-1 RNA from virions can be detected using this method and a positive signal could be detected with less than 50 pg of RNA from DEN-1 infected cells. No background signal was detected with a 10,000-fold excess of RNA from uninfected cells.

Sequence analysis of JEV and DEN-1 genomic cDNAs

Efforts to complete the sequencing of the DEN-1 genome were the primary responsiblity of two guest scientists, Dr. Zhang Yong-he and Dr. Xiao Ze-shuai. Drs. Zhang and Xiao have been able to sequence an additional 2.0 kb of the DEN-1 genomic cDNA, extending from approximately nucleotide 6,500 in the NS3 coding region to nucleotide 8,500 in the NS5 coding region. There are a number of minor discrepencies and gaps in this newly sequenced region that Drs. Zhang and Xiao were unable to resolve prior to their departure. Since their departure, our efforts to complete the sequencing of the DEN-

1 genome have continued at a reduced priority. Emphasis has been placed on resolving the minor discrepencies and gaps in the sequence completed by Drs. Zhang and Xiao and identifying new cDNA clones in our library that will allow us to complete the sequencing of the DEN-1 genome.

D. Future Work

Efforts for the furture will include:

1. Continued sequencing of the DEN-1 genome
2. Initiation of virus protein expression studies in animal cell systems.
3. Continued assessment of the immunological potential of recombinant proteins produced in the insect cell and *E. coli* systems.

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